Bemisia tabaci (Hemiptera: Aleyrodidae) nymphal feeding in cotton (*Gossypium hirsutum*) leaves

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Abstract We used brightfield electron microscopy (BEM), differential interference contrast microscopy (DICM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM) to investigate the stylet pathways of *Bemisia tabaci* during nymphal feeding behavior in cotton leaves beginning with penetration of the abaxial leaf surface and ending with stylets in sieve tubes in phloem tissues. Most nymphal stylets within salivary sheaths penetrating leaf tissues made complex turns and developed more than one salivary sheath branch before ending in sieve tubes. The external morphology of the salivary sheaths and their routes between and through leaf cells are described during the present study. Results showed the presence of the stylet within the sieve tubes. *B. tabaci* nymphs may remove stylets and feed in different sieve tubes. Ten short movies showing the progression of the stylet penetrations from adaxial surface to the sieve tubes are attached to Figures 8−15. The report and movies can be viewed from the internet. Download the movies to a local drive in your computer first for fast upload. The movies are posted on the website http://www.ars.usda.gov/Services/docs.htm?docid=14629. The movies can be used as a teaching aid in biology classes.

Key words *Bemisia tabaci*, microscopy, nymphs, feeding, stylet sheath, stylet
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Introduction

There have been three landmarks of upscaling of *Bemisia* from a trivial problem to a worldwide economic problem: (i) Sudan, middle east, and southwest of the USA in 1970−1979; (ii) Florida in 1986 (Gerling & Mayer, 1996), and (iii) USA in the early 1990s to the current years. *B. tabaci* (Gennadius) (Hemiptera: Aleyrodidae) biotype B has caused extensive economic damage in crop production worldwide (Bellows *et al.*, 1994; Birdsall *et al.*, 1995). The species has a wide range of crop (Basu, 1995) and weed hosts (Natwick *et al.*, 2000). *B. tabaci* are phloem feeders and may transmit viruses through the salivary canals of the stylets during feeding. *B. tabaci* are more likely to have higher feeding success events on cucurbits compared with lettuce, partially because the cucurbits have twice as many vascular bundles (Cohen *et al.*, 1998). Newly hatched nymphs (crawlers) can reach phloem tissue from any position on the abaxial leaf surface because their stylets may be as long as 113.8 ± 4.2 μm (Freeman *et al.*, 2001). Earlier studies indicated that some feeding by *Bemisia* nymphs was directed to non-vascular tissue (Cohen & Hendrix, 1994), but, in fact, all developed nymphs were found to connect with vascular tissues (Cohen *et al.*, 1996). Nymphs develop salivary sheaths that may serve to protect their stylets during leaf tissue entry of a host plant. The salivary sheaths leave a permanent record of stylet paths from the epidermis to the phloem tissue (Freeman *et al.*, 2006). Using a confocal laser scanning microscopy (CLSM), we

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showed that the salivary sheaths, upon contact with vascular bundles, sometimes encircle the xylem tubes before penetrating phloem tissues (Chu *et al*., 2001).

The objective of this study was to investigate the pathway of whitefly nymphal feeding in cotton leaves from the time of penetration of the abaxial leaf surface until the stylets enter the sieve tubes of phloem tissues. Salivary sheaths were used as a means of tracing stylet penetration. We include key CLSM photographs from an earlier report of *B. tabaci* nymphal feeding on cotton leaves (Chu *et al*., 2001) and photographs of the anatomy of a cotton leaf showing epidermis, mesophyll parenchyma tissue, sieve tubes of the phloem, and the interaction between the insect salivary sheath and the leaf tissue to provide a continuous progression of the feeding activity sequences from abaxial surface penetration to the sieve tubes.

Another objective of the current report is to summarize our recent reports on the *Bemisia tabaci* nymphal feeding on cotton (*Gossypium hirsutum* L.) into a series of photographs leading from a settled nymph on abaxial leaf surface to the feeding in sieve tubes in phloem tissues. One may download the information as a teaching aid in a biology class.

Materials and methods

This study used brightfield electron microscopy (BEM), differential interference contrast microscopy (DICM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and CLSM to investigate the stylet pathways of *Bemisia tabaci* during nymphal feeding behavior in cotton leaves. For the investigation using CLSM, greenhouse grown cotton, cv. D&PL 90, infested with *B. tabaci* nymphs was microscopically examined. Staining and clearing nymphs and leaves were accomplished following the procedures of Cohen *et al*. (1996) for whole mounts. The specimens were examined with the aid of a Leica TCS-4D upright CLSM with 488/568 dual excitation using a transmission detector with a 590 longpass filter. Magnifications of 50, 100, 200, and 400 × were used to trace the salivary sheaths throughout cotton leaf structures.

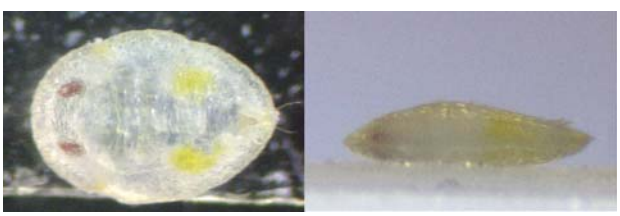

Fig. 1 A live fourth instar nymph on a clear cover glass. Top view (left) and side view (right).

Images were recorded, from 0.58 seconds to 10 μm optical sectioning intervals, from nymphal labium at the abaxial leaf surface to the vascular bundles. We used the NIH Image (NIH, Bethesda, MD, US) and VoxBLAS 3D reconstruction program (Vay Tek, Inc., Fairfield, IN, US) to view composite images of all focal planes containing stylet structures.

Results

A fourth instar, red-eyed nymph is shown in its live form position from top and side views (Fig. 1). First instar nymph (crawler) can move (Fig. 2 left) searching for feeding sites. A crawler is small relative to a red-eyed nymph (Fig. 2 right). Under a stereoscope, a cotton leaf shows a complex leaf vein network that consists of areoles with glands at their center (Fig. 3 left). The leaf surface has trichomes, areoles (delineated by the arrows), and a variety of cells with different shapes (Fig. 3 right). The areoles may vary in size in different cottons (Fig. 4 left and right) in a cotton leaf. Mesophyll leaf tissue is made up of the densely packed elongate palisade cells and loosely round spongy parenchyma cells (Fig. 5). Xylem tissues are positioned above phloem tissues in vascular bundles in leaves in the adaxial-abaxial orientations. The distance from lower epidermis to the phloem tissues is always shorter than the

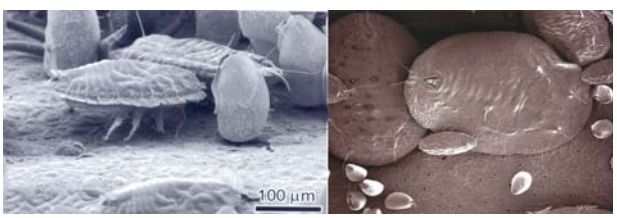

Fig. 2 A first instar nymph (left) and the size comparison of fourth and first instar nymphs (right).

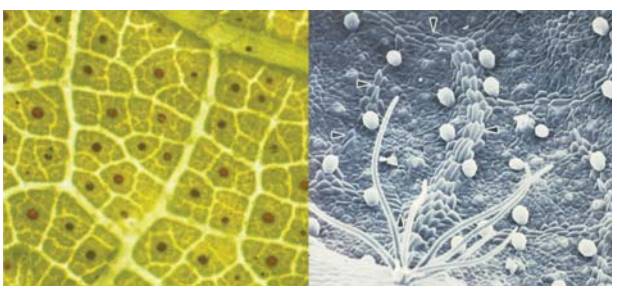

Fig. 3 Cotton leaf vein network with glands at center of an areole (left), and topographic lower leaf surface (right). An areole is delineated by arrows in the right photograph.

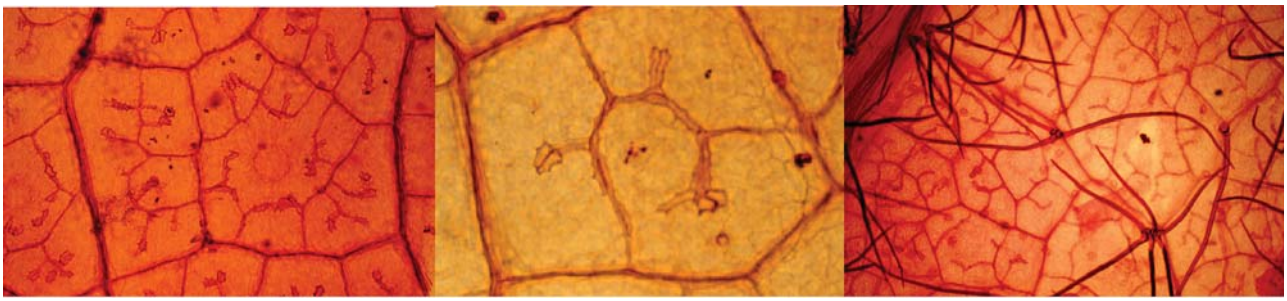

Fig. 4 Minor leaf veins in spongy mesophyll cells (left) and a higher magnification of a leaf areole (center) of a smooth cotton leaf, and a hairy cotton leaf with large trichomes (right). Note the areoles in the smooth leaf are larger compared with those in the hairy leaf.

distance from upper epidermis to the phloem tissues. Phloem tissue consists of sieve tubes (Fig. 6) and companion cells. Figure 7 shows the relative size of a large *B. tabaci* nymph and the depth of a cotton leaf. Figure 8 left and right show a salivary sheath wrapped around a vascular bundle. The nymph salivary sheath traveling through the leaf intercellular spaces and approaching a vascular bundle is shown in Figure 9 left, and after penetration in Figure 10. A high magnification of a salivary sheath top with a stylet tip can be seen in Figure 9 right. Figure 11 (left and right) shows a photograph series of a salivary sheath penetrating into the vascular bundle. Figure 12 (left and right) shows a salivary sheath in phloem tissue that appears parallel to the walls of sieve tubes. The nymphal salivary sheath can be traced around three sieve tubes in the phloem tissues (Fig. 13 left). The 3-dimentional (3-D) confocal photograph of the three branches of a salivary sheath from two different angles shows vividly the *B. tabaci* feeding pattern (Fig.

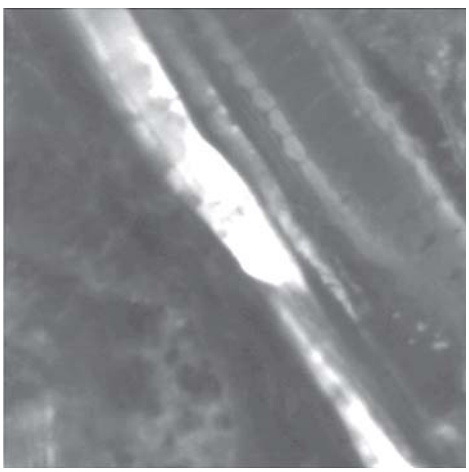

Fig. 6 Phloem tissue showing the sieve tube width from the brightened area to second diagonal line.

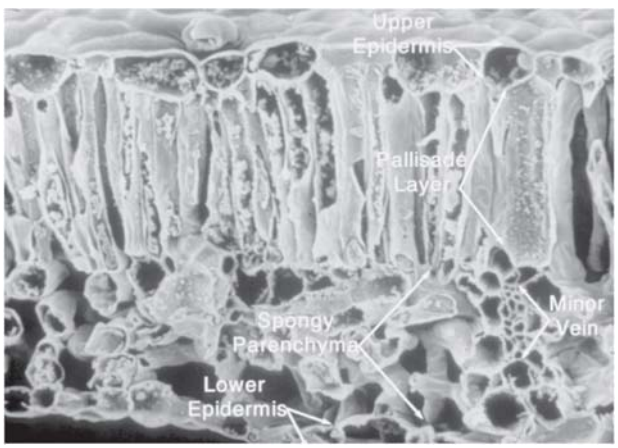

Fig. 5 A fractured cotton leaf showing leaf structure: closely packed single layer cells of upper epidermis, a block of thin-walled palisade mesophyll cells, and several layers of spongy mesophyll cells above a single cell layer of lower leaf epidermis.

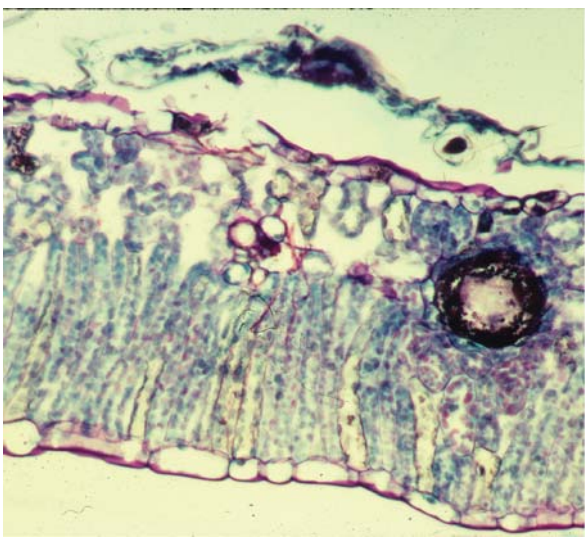

Fig. 7 A cross section of a *B. tabaci* nymph topped over the lower epidermis of a cotton leaf. Courtesy of Allen Cohen.

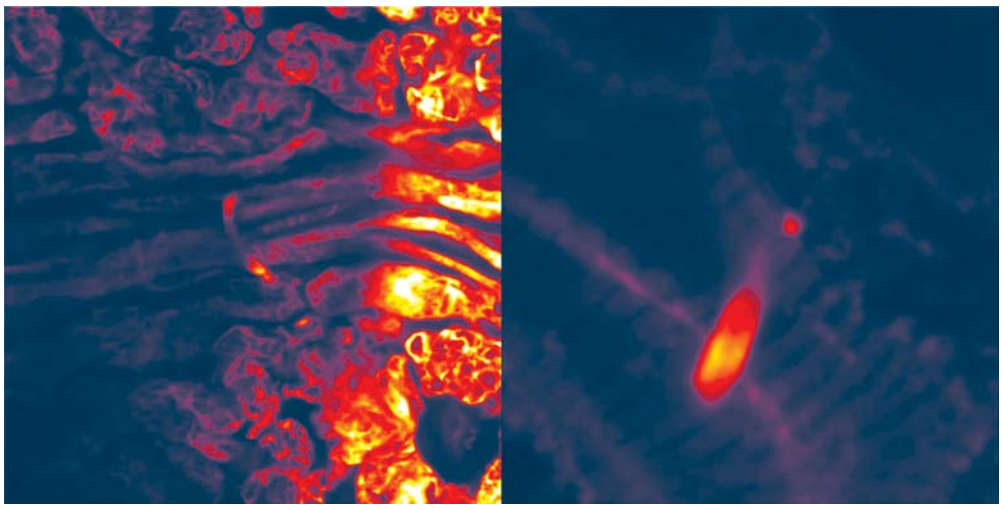

Fig. 8 *B. tabaci* crawler salivary sheath wraps around vascular bundles in a cotton leaf (left and right). Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Figs. 8A and 8B to view the progression of the salivary sheath.

Fig. 9 *B. tabaci* crawler salivary sheath (left) approaching a vascular bundle in a cotton leaf and on the right a higher magnification the tip of the salivary sheath showing the stylet. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 9 to view the progression of the salivary sheath.

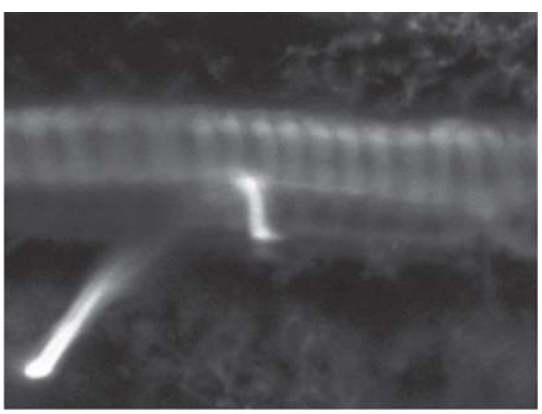

Fig. 10 *B. tabaci* crawler salivary sheath going into a vascular bundle in a cotton leaf. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 10 to view the progression of the salivary sheath.

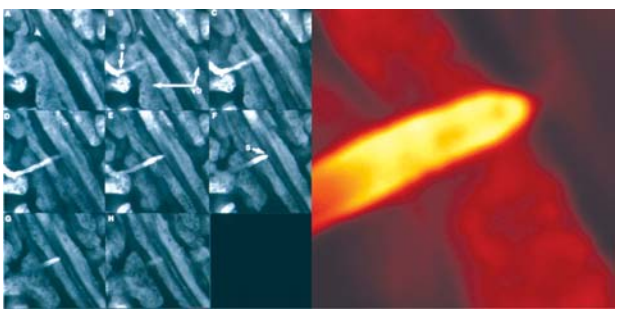

Fig. 11 A segmental series of a *B. tabaci* salivary sheath(s) traveling through a vascular bundle (vb) of a cotton leaf (left) and the enlarged tip of a salivary sheath. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Figs. 11A and 11B to view the progression of the salivary sheath tip.

13 center and right). A *B. tabaci* crawler stylet can be seen surrounded by the salivary sheath in the sieve tube in the left of Figure 14. The average widths ($\pm$ SD) are 4.2 $\pm$ 0.3 μm and 13.4 $\pm$ 1.2 μm (n = 10) for stylet and its sheath, respectively. In the left of Figure 15, a series of images shows a nymph from a cotton leaf surface into the leaf tissues. The 3-D confocal photographical reconstruction of Figure 15 at left is shown in Figure 15 at right.

Discussion

The search for feeding sites by *B. tabaci* crawlers appear to be random processes. Nymph stylets are long enough so that nymphs located on any part of leaves can tap into the nearest feeding site when entering through the leaf epider-

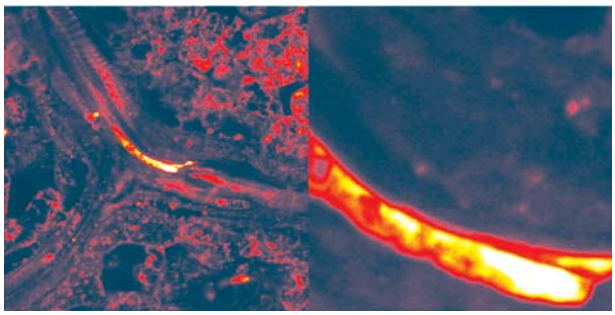

Fig. 12 A *B. tabaci* salivary sheath in phloem tissue of a vascular bundle (left) and a higher magnification of the sheath at the left. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 12 to view the progression of the salivary sheath that appears to parallel to a sieve tube in the phloem tissue.

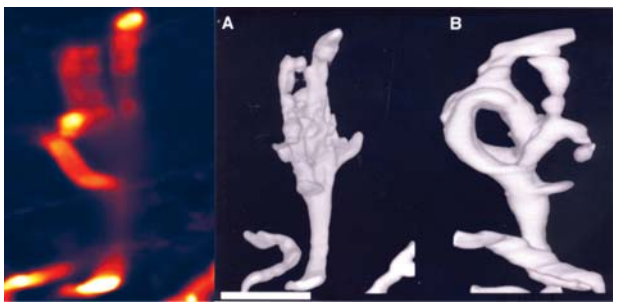

Fig. 13 *B. tabaci* nymphal salivary sheath penetration through an abaxial minor vascular bundle of a cotton leaf. The salivary sheaths (left) can be traced around three sieve tubes of a phloem tissue. A 3-dimentional reconstruction of the three salivary sheaths (center) and rotated 90° (right) shows the position of the sheath relative to the three sieve tubes of the phloem tissues. The salivary sheaths (left) can be traced around three sieve tubes of a phloem tissue. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 13 to view the progression of a single salivary sheath that branched out into three sheaths.

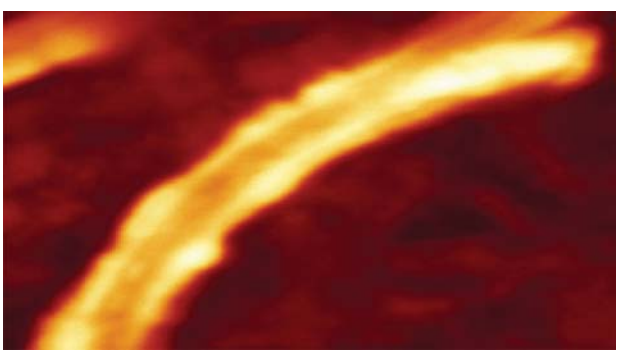

Fig. 14 A *B. tabaci* crawler stylet channel through which the stylet travels. Click here http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 14 to view the progression of a single stylet traveling through the sieve tube.

mis of abaxial leaf surfaces (Chu *et al*., 2001, Freeman *et al*., 2001). Most eggs, and nymphs, are found on abaxial leaf surfaces (Chu *et al*., 1995). *B. tabaci* adults may oviposit on adaxial cotton leaf surfaces; however, hatched crawlers in most cases move to abaxial leaf surfaces for habitat and feeding (Chu *et al*., 2004). *B. tabaci* nymphs usually feed on small veins in an areole (Fig. 4 center). The dense trichomes of hairy leaf cottons on abaxial leaf surfaces may provide a better protected habitat for nymphs from predators and, hence, are more attractive to *Bemisia* compared with smooth cottons (Fig. 4 right).

In leaf phloem tissues, sieve tubes are usually thick-walled in contrast to the thin-walled companion cells (Oparka & Turgeon, 1999). Nymph penetration through thick-walled sieve tubes instead of thin-walled companion cells suggests that nymphs may be able to differentiate turgor pressure of plant cells. Both sieve tubes and companion cells have solute potential much higher than surrounding cell types. The solutes in sieve tubes appear to flow into a nymph stylet without effort from the nymphs once stylets are in the sieve tube.

Stylet penetration through abaxial leaf surface epidermal cells searching for sieve tubes may provide an energy efficiency advantage for nymphs because of the shorter distance to sieve tubes from abaxial surface compared to the adaxial surfaces (Chu *et al*., 1995) and also because of less penetration resistance through loosely arranged spongy mesophyll cells compared to the compact palisade cells that are closely associated with adaxial leaf surfaces. Further, in consideration of the thickness of the salivary sheath that surrounds the stylet (Fig. 13) a nymph uses a great amount of salivary sheath material in searching for sieve tubes. Salivary sheaths have been shown to branch out to different directions during nymphal searching for feeding sites (Cohen *et al*., 1998; Chu *et al*., 2001). This appears to indicate that nymphs are either looking for an optimal sieve tube or changing from feeding in one sieve tube to the other tubes. The salivary sheath branches wrapped around vascular bundles may serve to anchor the stylet while searching and feeding into sieve tubes. The three rings of salivary sheaths in the phloem tissue in Figure 13 supports an earlier report of Cohen *et al*. (1998, Fig. 2A) that a minor vein may have three sieve tubes in association with a single xylem tube in phloem tissues.

The feeding behavior shown in the movies may be the first evidence available on the internet for a *B. tabaci* nymphal feeding in the sieve tubes of a cotton leaf. Feeding behavior by piercing-sucking plant-feeding insects involves much more complex plant-insect interactions than feeding by herbivores with chewing mouthparts (Walker, 2006). The series of events that guide *B. tabaci* to host plant selection, oviposition site selection, and nymphal feeding activity have been reported earlier (Chu *et al*., 1995; Cohen *et al*., 1996a,

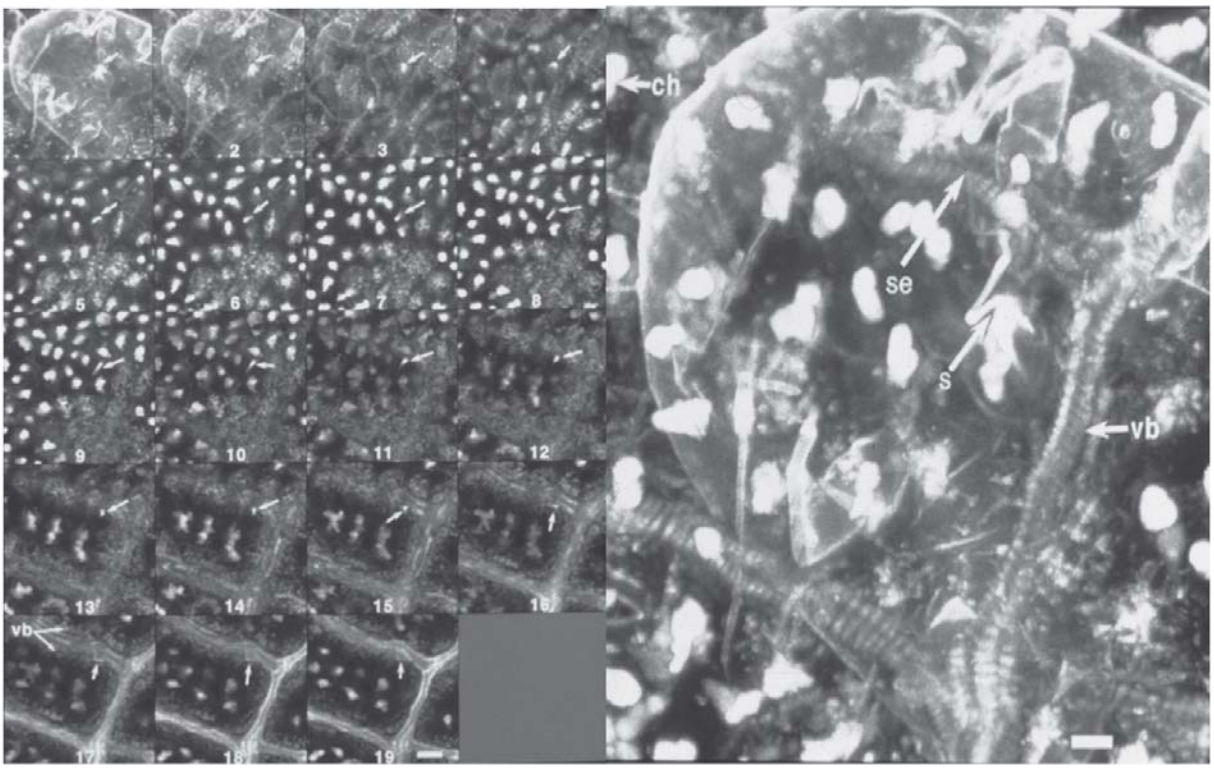

Fig. 15 A *B. tabaci* crawler on the abaxial cotton leaf surface. On the left, the arrows point to the nymph salivary sheath as it leaves the labial area and progresses through the leaf to the end at a vascular bundle (vb). On the right, a 3-dimensional reconstruction of the confocal series shown on the left. The salivary sheath (s) can be traced from the nymph through the leaf tissue to its end (se) in a vascular bundle (vb). Cells with aggregated chloroplasts (ch) appear as bright bodies throughout the leaf tissue. Click http://www.ars.usda.gov/Services/docs.htm?docid=14629 Fig. 15 to view the progression of the nymphal feeding from abaxial leaf surface to the sieve tubes.

b, 1998; Freeman *et al*., 2001). Although much progress has been made to define leaf stylet penetration and stylet pathway to phloem tissue, a complete understanding of chemical, physical and biological events in the process remain unknown. A complete understanding of *Bemisia* host selection and feeding behavior at the molecular level may lead to the development of control measures using innovative cultural practices, breeding resistant varieties, developing bio-insecticides, and better use of biological agents.

This report of *B. tabaci* nymphal feeding and its associated movies are posted on the website http://www.ars.usda.gov/Services/docs.htm?docid=14629. The movies can be used as a teaching aid in biology classes.

Acknowledgments

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